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TITLE: Biological Monitoring of HER-2 Positive Patients Using Serum HER-2 and Circulating Tumor Cells

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13. ABSTRACT *(Maximum 200 words)*

Purpose: Evaluate the feasibility of detecting CTCs by CK19 mRNA in patients with HER-2 positive metastatic breast cancer and correlate this with serum HER-2 and clinical response to Herceptin therapy.

Patients/Methods: Blood samples were collected from 55 women with HER-2 positive (IHC+++ and/or FISH+) metastatic breast cancer and 30 healthy female volunteers. Blood samples were collected at baseline, during with the first cycle of H&N at weeks 2, 3 and 5, and at each restaging. Peripheral blood mononuclear cells were isolated and enriched for epithelial cells by immunomagnetic selection. Plasma was used to measure shed HER-2 extracellular domain using as commercially available kit. The presence of CK19 gene mRNA was determined by RT-PCR using the LCx probe system (Abbott). Thirty healthy donor blood samples were accessed as controls.

Results: A total of 158 samples, from 24 patients were analyzed. Samples at baseline and first restaging were available in 23 patients, of which 11 had CK19 detectable (48%). CTC were only detected in patients with liver metastases (p=0.0004). All patients with CK 19 present at baseline responded to therapy. In subsequent cycles CK 19 remained negative in most patients. CK-19 mRNA was undetectable in 29/30 (97%) of healthy controls. In this study, 48/55 patients had serial samples available for analysis of shed HER-2. Of these, 24/47 (51%) had elevated ECD using a cut-off value of 20 ng/ml. Serum HER2 was correlated with visceral disease (p=0.018) and the percentage change was associated with disease progression. Comparison of serum HER-2 and CK-19 from the same patient show consistent expression patterns of shed HER-2 and CK-19.

Conclusion: Results of this study suggest that CTCs are detectable by RT-PCR for CK-19 and that this may identify a group of patients with metastatic liver disease. The pattern of CTC detection mirrors that of circulating HER-2 and their behavior is consistent with clinical response to Herceptin therapy.

14. SUBJECT TERMS
HER-2 extracellular domain, circulating tumor cells, metastatic breast cancer, Herceptin, Navelbine

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Table of Contents

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U.S. Army Medical Research Acquisition Activity
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Project title: Biological Monitoring of HER2 Positive Patients Using Serum HER2 and Circulating Tumor Cells
Principal investigator: Cinara Dias, M.D., M.Sc.
Mentor: Lyndsay Harris, M.D.

ANNUAL SUMMARY

The report shall contain a cover Standard form (SF 298) and table of contents.
The content must include
1) A bulleted list of key accomplishments;
2) A list of reportable outcomes that have resulted from this award to include: manuscripts, abstracts, degrees, tissue or serum repositories.

Aim 1: To evaluate the utility of measuring circulating HER2 Extracellular Domain (ECD) in Metastatic Breast Cancer Patients in monitoring Response and Progression of Herceptin® and Chemotherapy.

a) Organize the collection of serum from patients enrolled in "Herceptin®/Navelbine Multicenter Clinical Trial".
b) Perform ECD ELISA on batched sets of 40 samples with appropriate controls (estimate sample number = 250 samples per year).

For this part of the proposal Dr. Dias has worked closely with the data managers in Dr. Harris' laboratory and the study coordinators in the breast cancer clinic to ensure that samples were collected, processed and shipped appropriately.

The following has been accomplished since the inception of the proposal:

- 55 patients from 16 medical institutions have received 378 cycles of therapy.
- 278 blood samples have been collected on these 55 patients (table 1).
- 4 patients refused consent for blood collection on the study.
- Plasma from 39 healthy female donors from the DFCI were collected, processed and stored for later analysis.

Table 1. Patients-samples data.

	DFCI	Other institutions	Total
Patients (pt)	17	38	55
Samples	123 (7/pt)	155 (4/pt)	278 (5/pt)
Cycles	138 (8/pt)	240 (6/pt)	378 (7/pt)
Samples collected	123 (89%)	155 (64.5%)	278 (73.5%)
Samples not collected	15	85	100

Data between parentheses refer to averages or percentages.

Dr. Dias supervises the technical staff in Dr. Harris' laboratory who are responsible for the collection and processing of the blood samples from patients enrolled in the "Herceptin®/Navelbine Multicenter Clinical Trial". Collection of blood for circulating tumor cells is performed in conjunction with plasma using a special phlebotomy tube (cell preparation tube - CPT) that allows both biospecimens to be obtained from one blood sample.

The following procedures are in place for this part of the proposal:

- Samples are processed immediately after arrival starting with a separation step, which isolates the blood cells from the plasma using a density gradient method. The plasma is collected and frozen. The buffy coat (which contains the white blood cells and the tumor cells) is washed and frozen in a rate-controlling freezer, in order to preserve cell viability.
- In this first cohort of patients, three institutions processed the blood samples themselves and sent the frozen plasma and tumor cells after the patients completed the study. As the number of samples received from other institutions was only 65%, we put into place a system where the participating institutions send the CPT tubes with the blood to be processed in the DFCI.
- To date, 73.5% of the samples are collected on the expected dates as determined by the protocol. A tracking system involving anticipatory phone calls to the medical centers was implemented in order to assure the retrieval of samples as scheduled and the processing within 24 hours of collection. Previous experiments performed in the laboratory have demonstrated that sample preservation is maintained up to 24 hours after collection.

Under the supervision of Dr. Dias, shed HER2 extracellular domain was measured using a commercially available kit (HER2/neu ELISA®; Oncogene Science). Samples to be analyzed were processed in triplicate with 5 internal controls to plot a standard curve in order to quantify the levels of HER2/ECD in the plasma. In order to protect the confidentiality of patients and integrity of the study, the data manager records information on patient samples while the study coordinators store clinical information in a separate research database. Only after biomarker measurement these datasets were merged for statistical analysis. Preliminary data were generated by comparing rate of positivity of HER2 ECD with disease characteristics and response to therapy.

The following results were seen in the first patient cohort:

- Plasma HER2/ECD was measured in 47/55 patients with available serial samples.
- 24/47 (51%) had elevated HER2/ECD using a cut-off value of 20 ng/ml.

- HER2/ECD was correlated with visceral disease (p=0.018), consistent with published observations.
- ECD levels declined in both responding and progressing patients, possibly due to the fact that Herceptin® binds to the ECD and increases its clearance from the circulation. Herceptin® is described to inhibit constitutive HER2 cleavage and/or shedding and this may correlate with the clinical anticancer activity of the multifunctional HER2-targeting antibody (Baselga J, et al. Semin Oncol 2001 Oct;28(5 Suppl 16):4-11).
- Statistical analysis revealed that the percentage change between baseline and restaging predicted disease progression (figure 1).

Aim 2: To compare methods of measuring HER2 in Breast Cancer Tissue

a) Coordinate retrieval of paraffin-embedded primary tumor tissue for patients enrolled in the "Herceptin®/Navelbine Multicenter Clinical Trial" at DFCI and participating institutions by working with research coordinators/data managers to review pathology reports, select appropriate blocks and request blocks or 4um unstained slides from the institution where the original surgery was performed. Assure that all data regarding tumor blocks is recorded in research database.
b) Perform immunohistochemistry and fluorescence in-situ hybridization on tumor tissue per methods outlined in proposal. N=75 cases/year.

Dr. Dias oversees a research coordinator in Dr. Harris' laboratory who contacts the appropriate institution in order to retrieve paraffin-embedded primary tumor tissue and/or unstained slides from patients enrolled in this clinical trial. The process for obtaining blocks and slides has involved an amendment to the original protocol (which did not request tissue for research studies) and has required reconsenting of patients to allow tissue collection. As this is a multicenter study, it is often necessary to work with data managers in all the participating institutions to send letters to patients and retrieve pathology material. This has been a fairly labor intensive process; therefore, our progress has been somewhat slower than anticipated for this aim.
The following accomplishments have been achieved:

- Retrieval of tumor tissue from 18 patients: paraffin embedded blocks (4) and unstained slides (14).
- Four additional patients have agreed to allow the study of their tissue and requests were submitted to the pathology department for tissue retrieval.
- Seven samples are irretrievable, as the biopsy specimens are either missing (5) or the patients refused consent (2).
- Two participating institutions are in the process of review of the protocol amendment by their Internal Review Boards.

We anticipate the cohort will be collected by the end of this year at which time the assays will be performed on these samples. Dr. Harris' laboratory has expertise with all the assays described in the proposal and we do not anticipate any difficulty completing the study once tissue is collected.

Aim 3: To detect and monitor the molecular phenotype of circulating tumor cells (CTC) in HER2 Positive Metastatic Breast Cancer patients treated with Herceptin® and Chemotherapy.

a) Organize the collection of peripheral blood samples from patients enrolled in "Herceptin®/Navelbine Multicenter Clinical Trial" at DFCI and selected institutions by working with research coordinators/data managers to ensure that samples are collected on time, processed and shipped appropriately and data is recorded research database.
b) Perform bead extraction and RT-PCR for cytokeratin, BU-101 and HER2 on CTC samples per methods outlined in proposal (N=100 samples/year).

Dr. Dias supervises the collection of blood for circulating tumor cells, which is performed in conjunction with plasma collection.

Positive selection of epithelial cells from the blood is performed using immunomagnetic beads (Dynabeads®) coated with antibodies for CK-19 (a cytokeratin). Tumor cells from blood samples have their mRNA extracted using a commercially available kit from Qiagen®. As all epithelial cells express cytokeratins and the majority of breast cancer cells seem to express CK19, this is considered to be a useful marker for detection breast epithelial cells in peripheral blood.
The following experiments were performed in our model system to establish sensitivity and specificity of detection of circulating tumor cells using CK-19 and HER2 measured by RT-PCR from the peripheral blood:

- ✓ The expression of CK-19 and Beta 2 microglobulin (B2MG – a "house-keeping" gene used as an internal control) in circulating tumor cells was evaluated using the Abbott LCx® Probe System. In order to determine the detection level of tumor cells in clinical samples, breast cancer cells were spiked in blood samples from healthy volunteers. The LCx Probe System has a reproducible sensitivity of 10 tumor cells per 8 ml blood sample (figure 2).

  Preliminary data from the first cohort of patients is as follows:
  - Measurement of CK19 mRNA in peripheral blood was performed using the LCx Probe System in 158 samples from 24 patients with available serial samples and 30 healthy female volunteers.
  - Samples at baseline and first restaging are available in 23 patients, of which 11 (48%) had CK 19 detectable.
  - CK-19 mRNA was undetectable in 29/30 (97%) of healthy controls
  - CTC were only detected in patients with liver metastases (p=0.0004).
  - All patients with CK 19 present at baseline responded to therapy
  - In subsequent cycles CK 19 remained negative in all the patients who had it detectable at baseline.
  - In two cases, low positive values were detectable prior to clinical progression.
  - The comparison of serum HER2 and CK-19 from the same patient showed similar expression patterns of both.

- The preliminary results of this study suggest that CTC are detectable by RT-PCR for CK-19 and this may identify a group of patients with metastatic liver disease. The pattern of CTC detection mirrors that of circulating HER2 and that their behavior is consistent with clinical response to Herceptin® therapy.

✓ As the LCx system is a semi quantitative method, we developed techniques to assess the expression of HER2 and B2MG using quantitative PCR (qPCR) with TaqMan®. With this procedure, the sensitivity decreased to 100 cells per 8 ml of blood. Therefore, we developed a more sensitive method using specially designed primers (Molecular Beacons®). This one-step qPCR technique yielded the detection of 10 tumor cells expressing HER2 in an 8ml blood sample (figure 3).

✓ We further designed a set of primers for CK19 using the Molecular Beacons® system. The use of Molecular Beacons® proved to be very sensitive and allowed the detection of down to 0.5 cell equivalent expressing CK19 (figure 4).

Further studies will focus on a larger patient cohort to confirm or refute these findings. In addition, we plan to use the quantitative and more sensitive Molecular Beacons technology to measure expression levels of the genes B2MG, BU-101 and HER2 in blood from volunteers spiked with breast cancer cell lines first, followed by the analysis of patient samples.

Reportable outcomes that have resulted from this award include:

- Abstract selected for oral presentation at the XXXth Meeting of the International Society for Oncodevelopmental Biology and Medicine – Translational Cancer Research. Sept 2002. Boston, USA.
- This first cohort of 55 patients allowed tissue (18 samples), tumor cells (24 samples) and serum (278 samples) to be collected, organized and stored for future analysis and to became part of our repositories.

List of tables:

List of figures:

Figure 1. ROC analysis. The percentage change HER2/ECD from baseline to cycle 1 predicts progression of disease.

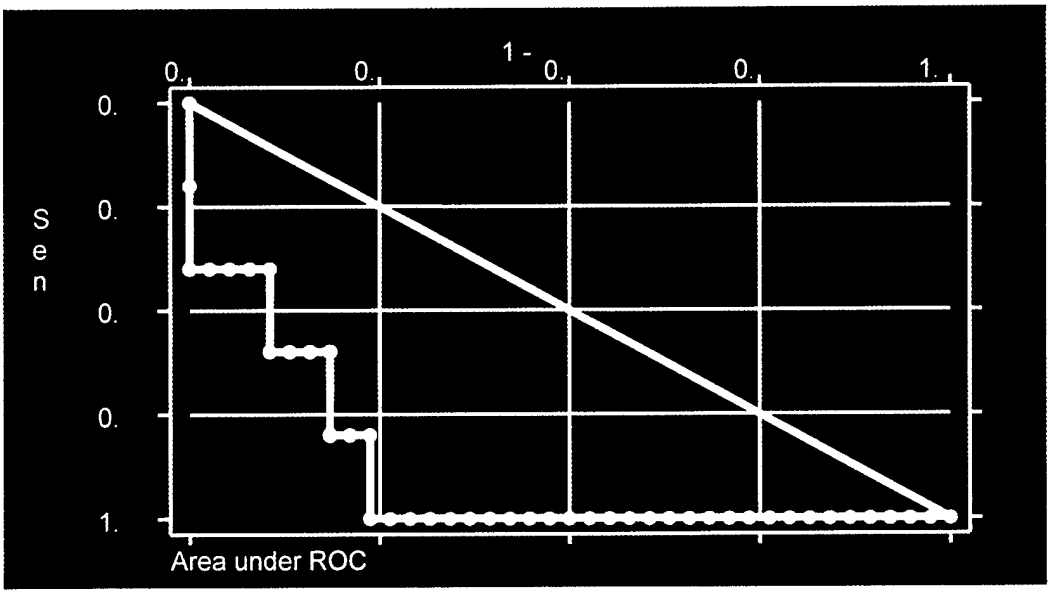
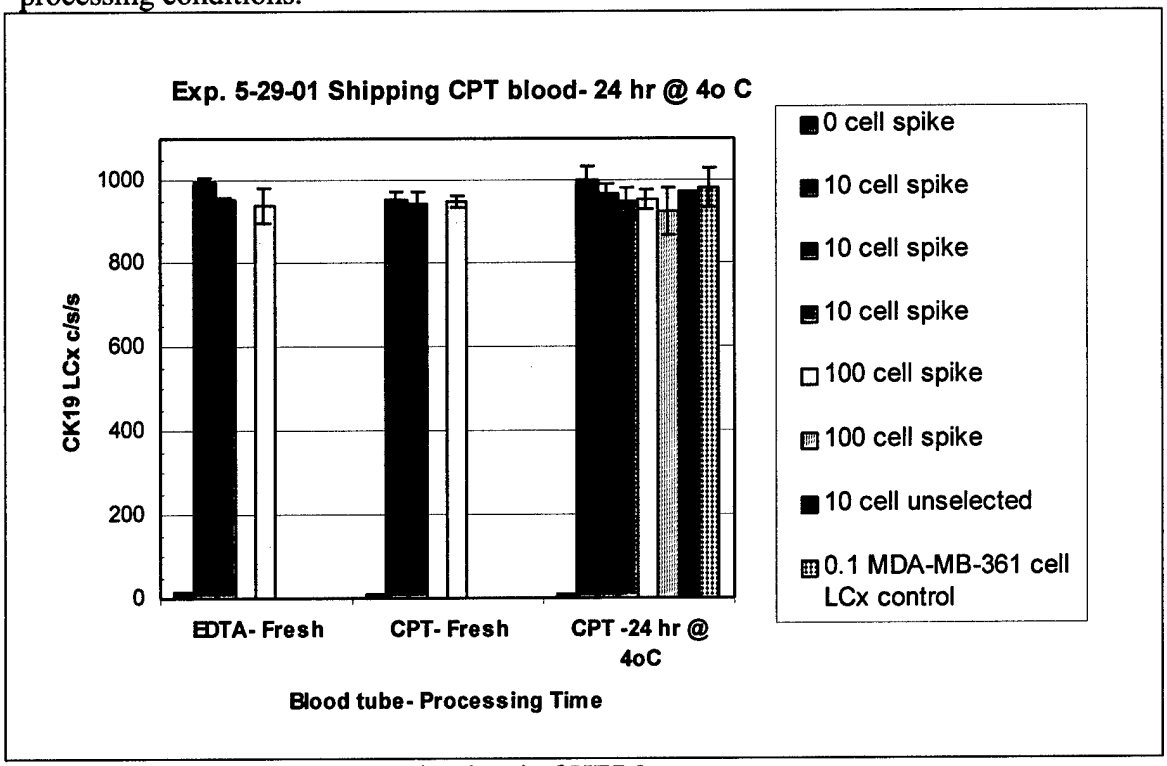
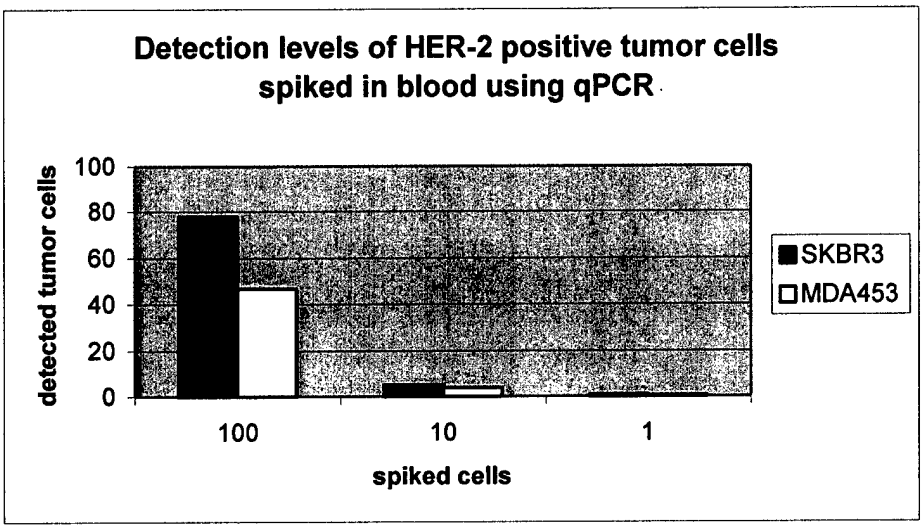


Figure 2. CK19 expression in blood samples spiked with MDA361 (HER2 positive breast cancer cell line) cells using the LCx system. Tumor cell detection under different processing conditions.



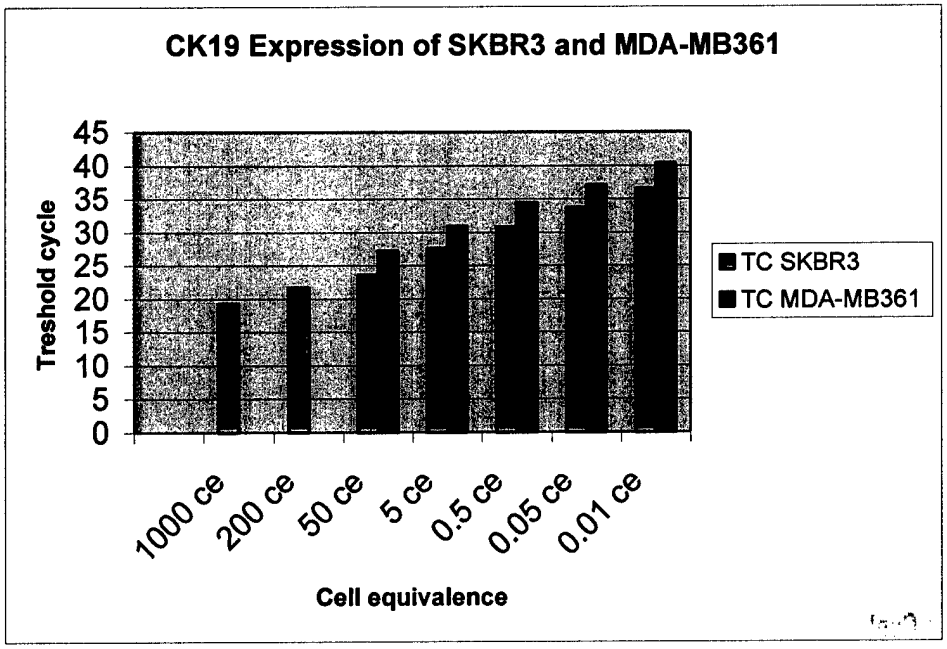
MDA361: intermediate expression level of HER2

Figure 3: HER2 expression in blood samples spiked with two HER2 positive breast cancer cell lines (SKBR3 and MDA453) using one-step qPCR with designed primers and probe.



SKBR3: high expression level of HER2
MDA 453: intermediate expression level of HER2

Figure 4: CK19 expression in blood samples spiked with two HER2 positive breast cancer cell lines using Molecular Beacons®.



MDA361: intermediate expression level of HER2
SKBR3: high expression level of HER2